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# Interactive effects of global warming and eutrophication on a fast-growing Mediterranean seagrass

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ABSTRACT

Coastal ecosystems, such as seagrasses, are subjected to local (e.g. eutrophication) and global (e.g. warming) stressors. While the separate effects of warming and eutrophication on seagrasses are relatively well known, their joint effects remain largely unstudied. In order to fill this gap, and using *Cymodocea nodosa* as a model species, we assessed the joint effects of warming (three temperatures, 20 °C, 30 °C and 35 °C) with two potential outcomes of eutrophication: (i) increase in nutrients concentration in the water column (30 and 300 μM), and (ii) organic enrichment in the sediment). Our results confirm that temperature in isolation clearly affects plant performance; while plants exposed to 30 °C performed better than control plants, plants exposed to 35 °C showed clear symptoms of deterioration (e.g. decline of photosynthetic capacity, increase of incidence of necrotic tissue). Plants were unaffected by high ammonium concentrations; however, organic enrichment of sediment had deleterious effects on plant function (photosynthesis, growth, demographic balance). Interestingly, these negative effects were exacerbated by increased temperature.

Our findings indicate that in addition to the possibility of the persistence of *C. nodosa* being directly jeopardized by temperature increase, the joint effects of warming and eutrophication may further curtail its survival. This should be taken into consideration in both predictions of climate change consequences and in local planning.

## 1. Introduction

Coastal ecosystems are facing multiple anthropogenic stressors that adversely affect their biodiversity and functioning (Vinebrooke et al., 2004). Such stressors are generated at a range of spatial scales, from the global to the most local. Global stressors are mostly related to climate change and include rising sea level, seawater acidification, warming and an increased frequency of heat waves (IPCC, 2013). The most prominent and pervasive stressor generated locally is probably eutrophication: increased loading of nutrients and organic matter from human activities (Nixon, 2009). This has come to be considered one of the major threats confronting coastal ecosystems (Bricker et al., 2008; Hemminga and Duarte, 2002). The knowledge accumulated to date on the effects of individual stressors on key species is impressive. However, stressors rarely occur in isolation in the environment and, when acting together, they can be synergistic, additive or antagonistic (Todgham and Stillman, 2013). The interaction between stressors is now viewed as a crucial issue, to the point that it is recognized that single-factor experiments are of limited use for assessing the effects of climate change on coastal marine ecosystems subjected to other disturbances, such as eutrophication (Wernberg et al., 2012). Undoubtedly, experiments with single stressors can help us gain knowledge of the intrinsic, basic response mechanisms involved. However, the results should only be extrapolated to nature with great caution, not only due to problems associated with scaling up, but also due to potential interactions with concurrent stressors (Gunderson et al., 2016).

Seagrasses are widespread habitat-forming species of great ecological value that are exposed to multiple threats and are currently suffering declines worldwide (Waycott et al., 2009). The effects of climate change (including increased temperature and acidification) or eutrophication on the distribution, abundance and vitality of seagrasses are relatively well known (see reviews by Koch et al., 2013, for climate change; and Burkholder et al., 2007, for eutrophication), and even though their effects have, for the most part, been assessed separately

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(but see Campbell and Fourqurean, 2014, 2018; Ow et al., 2016). Thus, it is well known that eutrophication has two main consequences for seagrasses. On the one hand, the effects of increased nutrient concentrations are generally considered detrimental, although they strongly depend on species-specific features and on local conditions (Kilminster et al., 2015; Romero et al., 2006; Ruiz et al., 2001). Thus, while a moderate supply of nutrients to plants adapted to nutrient-poor environments can stimulate growth (Alcoverro et al., 1997; Pérez et al., 1991; Short, 1987), once a threshold is reached, it may cause negative effects on plant photosynthesis and may even curtail survival (Brun et al., 2002, 2008; Hauxwell et al., 2003; van Der Heide et al., 2008). These negative effects can be caused directly, mainly by ammonium toxicity (Touchette and Burkholder, 2000; Van Katwijk et al., 1997); or indirectly, by stimulating phytoplanktonic, epiphytic and macroalgal overgrowth, and enhancing negative biotic interactions such as macro herbivore activity (Campbell et al., 2018; Ruiz et al., 2009; Wear et al., 1999). On the other hand, an increased supply of organic matter to the seagrass sediment, such as that caused by eutrophication, stimulates its oxygen demand, eventually leading to hypoxic or anoxic conditions (Frederiksen et al., 2008; Pérez et al., 2007). This oxygen shortage not only blocks metabolic function in seagrass roots, including respiration, growth and nutrient acquisition (Smith and Piedrahita, 1988), but it also stimulates microbial sulphate reduction, which leads to below-ground seagrass organs (rhizomes and, specially, roots) being exposed to sulphide, a strong phytotoxin (Holmer and Bondgaard, 2001). Despite seagrass having evolved a number of adaptations which increase its chances of surviving in naturally organic-rich sediments (Hasler-Sheetal and Holmer, 2015), additional deposition of organic C can exceed the seagrass response capacity, and have negative effects such as reduced photosynthesis, impaired growth or, in some cases, mass mortality (Collier and Waycott, 2014; Frederiksen et al., 2008; Koch et al., 2007; Olivé et al., 2009).

Temperature affects seagrass physiology in a number of ways. It is known that increased temperature usually stimulates both photosynthesis (Campbell et al., 2006; Winters et al., 2011) and respiration (Schulze et al, 2005); but beyond some threshold, it generally increases the latter more than the former, thus leading to an impaired C balance and reduced growth (Lee et al., 2005, 2007; Marín-Guirao et al., 2016, 2018; Pérez and Romero, 1992). Temperature also affects other processes, such as for instance nutrient uptake (Borum et al., 2004; Bulthuis, 1987) or protein synthesis (Campbell et al., 2006; Marín-Guirao et al., 2017). Overall, when the temperature exceeds a given threshold, which is largely species specific, thermal stress leads to a reduction in growth (Lee et al., 2007), deterioration of shoot status and eventually shoot mortality (Marbà and Duarte, 2010). The responses of seagrasses to increased temperature are relatively well documented; however, little is known of the potential distortion of these responses caused by eutrophication.

Global warming is expected to increase in the coming decades and will affect the surface waters of almost all of the world's oceans. Meanwhile, a large part of the planet's coastal areas are subjected to different degrees of eutrophication (Halpern et al., 2007), which is especially notable in industrialized countries. Consequently, many cases, thermal stress will have an impact on meadows already affected by chronic or acute eutrophication, whose responses to thermal stress will probably differ from that of unaffected plants, thus limiting our ability to make reliable and realistic predictions for future warming scenarios. To date, only a few studies have focused on the combined effects of warming and other stressors, such as anoxia (Koch et al., 2007, with *Halodule wrightii* and *Thalassia testudinum*), nutrients (Kaldy, 2014, with *Zostera marina*) or light (York et al., 2013 with *Zostera muelleri*). These works seem to suggest that synergistic effects are more the rule than the exception. If this is the case, the consequences of global warming may be worse than expected based solely on studies of thermal effects. In fact, a synergistic interaction between eutrophication and seawater warming has already been suggested for the Mediterranean seagrass *Posidonia oceanica* to forecast trajectories in abundance and distribution of this seagrass species in the context of the different global climate change scenarios (Jordà et al., 2012). However, a considerable gap exists in our knowledge of the combined effects of warming and other stressors; and research is needed to confirm (or refute) the potential synergies in seagrasses, especially in species that dominate areas that are particularly sensitive to climate change. The present study attempts to help fill this gap, by evaluating the joint effect of warming and eutrophication on a Mediterranean seagrass (*Cymodocea nodosa*). The Mediterranean is one of the regions that are expected to be most affected by warming, and the sea surface temperature rise, already in evidence (Burrows et al., 2011; Jordà et al., 2013) may reach 3 °C by the end of the 21st century (Jordà et al., 2012); while the frequency of heat waves is also expected to increase (IPCC, 2013). Moreover, eutrophication has been identified as one of the major environmental threats to seagrass habitats in coastal areas, mainly due to loading from urban, agricultural and aquaculture wastes, particularly in the more confined environments where *C. nodosa* is dominant (Boudouresque et al., 2009).

*C. nodosa* is widely distributed across a broad variety of shallow Mediterranean environments, from open coastal areas to coastal lagoons, and extends into the Atlantic, from the south of the Iberian Peninsula to the Canary Islands and Mauritania (Green and Short, 2003; Mascaró et al., 2009; Reyes et al., 1995). Its ecological value and its capacity to survive relatively eutrophic conditions (Oliva et al., 2012), as well as its considerable phenotypic plasticity (Pérez and Romero, 1994; Sandoval-Gil et al., 2014), make it an interesting model species to evaluate the joint effects of increased temperatures and eutrophication.

The aim of this study is thus to explore the combined effect of a global stressor (warming) and a local stressor (eutrophication) on functional traits of *C. nodosa*. We partition the eutrophication effects into an increased nutrient concentration in the water column and an increase of organic matter loading of the sediment. We then determine the response of the plant to each one of the three stressors (elevated temperature, nutrient increase and increased organic matter loading) separately; and also to the combined effects of temperature and each of the other two. The main hypothesis we wish to evaluate is that temperature and eutrophication act synergistically, with deleterious consequences for the seagrass. To this end, we perform two fully factorial experiments on indoor mesocosms in which plants are exposed to three levels of temperature and, on the one hand, to three different nutrient concentrations and, on the other hand, to two different levels of organic matter in the sediment.

## 2. Material and methods

We explored the interactive effects of eutrophication and temperature in two separate experiments. In the first experiment (TNUT experiment, hereinafter), temperature increase and nutrient (ammonium) addition were applied; while in the second (TANOX experiment, hereinafter) the stressors were temperature increase and addition of labile organic C to the sediment.

### 2.1. Plant and sediment collection

Undamaged healthy *C. nodosa* shoots (including their rhizomes and roots) were carefully collected by hand from a shallow, undisturbed meadow (0.5 m deep) in Alfacs Bay (NW Mediterranean) in late April. Only shoots less than one year old (less than 12 scars on the vertical rhizome, Mascaró et al., 2014) were selected to reduce the effects of physiological and morphological variability between shoots of different ages (Pagès et al., 2010; Pérez and Romero, 1994). Sediment was collected from the same area, extracting the surface layer (up to 10 cm deep), and immediately sieved (1 mm pore) to exclude macroinvertebrates and detritus. Sediment and plants were then transported separately in aerated tanks to the laboratory, where they were

maintained with aeration for one night prior to the experiment being setup. Temperature was kept constant at the ambient values measured at the collection site (19.5 °C). The experiments were conducted at the Experimental Chambers Service of the University of Barcelona.

### 2.2. Experimental design and setup

Both experiments were conducted using cylindrical transparent aquaria (12 L capacity, 40 cm height x 20 cm diameter) placed randomly in 3 experimental chambers (2.1 $m^2$). Each aquarium had an independent air pump providing proper aeration. The chambers allowed us to control the water temperature (20 °C, 30 °C and 35 °C) and incident light (270 μmol photons $m^{-2}$ $s^{-1}$), which was above the saturation irradiance for these plants (Pérez and Romero, 1992) on a 12 h:12 h light:dark photoperiod. To avoid experimental bias and minimize any uncontrolled variability, the aquaria were randomly relocated within the chambers every two days. Moreover, the aquaria were moved from one chamber to another (changing the chamber temperature) so that they spent approximately 1/3 of the experimental period in each chamber. Within 24 h of collection, twenty shoots (with their corresponding portion of rhizome and roots) were planted in each aquarium, previously filled with 10 cm of sediment and 9 L of filtered seawater. All the aquaria were covered with plastic film to prevent water evaporation. For the TNUT experiment, a total of 27 aquaria were prepared and distributed randomly in groups of 9 in the three experimental chambers; while for the TANOX experiment, a total of 18 aquaria were distributed randomly in groups of 6 (see experimental setting in Fig. 1).

The aquaria were kept at 20 °C for four days (a temperature close to that registered during sampling) to allow for plant acclimation. After the acclimation period, the temperature in two chambers was increased progressively (ca. 3 °C per day) until reaching 30 °C in one and 35 °C in the other. The third chamber was left at 20 °C as a control. The choice of the experimental temperatures was based on an unpublished 3-year temperature data series collected by the authors using continuous *in situ* recorders, indicating average summer (July to September) temperatures close to 30 °C, but peaking to 33 °C in the hottest summer. Based on this, we consider that 35 °C during heat waves is a reasonable assumption under a climate change scenario (IPCC, 2013).

For the TNUT experiment, once the experimental temperatures were reached, $NH_4Cl$ was added to obtain the following concentrations: control (no $NH_4Cl$ added), moderate concentration (30 μM of $NH_4Cl$) and high concentration (300 μM of $NH_4Cl$). The so-called "moderate" value (30 μM) can be observed at eutrophic sites and can trigger responses in some seagrass species (Villazán et al., 2013). The "high" value (300 μM) represents extreme events and would only be reached during some wastewater discharges (Cabaço et al., 2013). To counterbalance plant uptake (roughly estimated from growth requirements, Pérez and Romero, 1994), $NH_4Cl$ was further added to the experimental aquaria every 5 days: 3.8 mg of $NH_4Cl$ to the moderate ammonium treatment aquaria and 7.6 mg $NH_4Cl$ to the high ammonium treatment aquaria (with a total of two pulses after the initial addition at the beginning of the experiment). Each ammonium treatment was applied to three aquaria, chosen at random, within each temperature chamber, resulting in a complete factorial design with n = 3 replicates per experimental condition. The exposure period to both factors lasted 15 days, after which time leaves in the high-temperature treatment (35 °C) began to show critical necrosis marks.

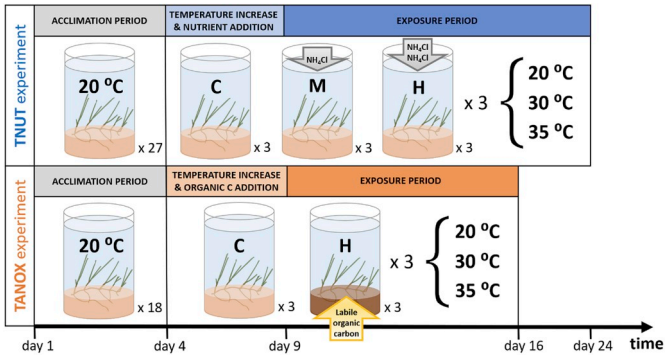


**Fig. 1.** Experimental setting. Grey arrows indicate ammonium addition in water and yellow arrow indicates labile organic carbon addition to sediment. TREATMENTS: C, control; M, moderate and H, high (see text). Temporal axis indicates: day 1, the beginning of the experimental setup with acclimation at control temperature; day 4, end of acclimation period and progressive increase of temperature; day 9, nutrient or labile organic carbon addition and start of the exposure period; day 16, end of TANOX experiment (7 days exposition); day 24, end of TNUT experiment (15 days exposition). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

For the TANOX experiment, once the experimental temperature was reached, the organic matter treatments were applied by adding labile organic C in the form of sucrose to the sediment in the aquaria as follows: control (no sucrose addition, 0.7% DW sediment C content in natural conditions) and high (675 g of sucrose added ≈ 15% DW sediment C content). The labile organic C treatments were applied to three aquaria, chosen at random, within each temperature chamber, resulting in a complete factorial design with n = 3 replicates per experimental condition. The experiment ended after 7 days of exposure period, when leaves in the high-temperature treatment (35 °C) began to show critical necrosis marks.

### 2.3. Water and sediment analysis

The concentration of ammonium in the water was analysed in each aquarium at the beginning and end of the TNUT experiment using an FP 2020 Plus fluorometer and following a standard method (Kérouel and Aminot, 1997). The redox potential of the sediment was measured at the end of the TANOX experiment using a Thermo Scientific, Orion Star A211 electrode. Measurements were taken in the upper 5 cm of the sediment layer.

### 2.4. Plant biochemical composition

To verify that the additions of ammonium and labile organic C applied could affect plant conditions, directly (high level of nutrients) and indirectly (anoxic sediment), we determined the N content (TNUT experiment) and S content (TANOX experiment) of different plant parts. To do this, at the end of the experiments, all the remaining shoots were harvested from the aquaria and separated into leaves, rhizomes, and roots. Subsequently, all plant tissues were dried at 60 °C and then finely ground and homogenized; finally, they were weighed and packed into tin microcapsules.

For the TNUT experiment, the nitrogen content of leaves, rhizomes and roots was measured using a Carlo-Erba elemental auto-analyser. For the TANOX experiment, vanadium pentoxide was added, and the sulphur content of leaves, rhizomes and roots was determined. Samples were analysed at the Scientific and Technological Centre (CCiT) of the University of Barcelona.

### 2.5. Measurement of plant traits

The plant responses to the different stressors (or their combination) were assessed via measurement of a series of traits, from the physiological to population level.

These included maximum quantum yield of PSII ($F_v/F_m$), incidence of leaf necrosis, leaf growth, rhizome elongation, and shoot demographic balance. All these variables have previously been used in the assessment of seagrass responses to stress and are related to plant health and performance (Beer et al., 1998; Frederiksen et al., 2008; Maxwell

and Johnson, 2000; Pagès et al., 2010; Romero et al., 2007). Rhizome elongation was only determined for the TANOX experiment, while all the other traits were measured in both experiments.

Maximum quantum yield of PSII ($F_v/F_m$) was determined using a diving PAM (pulse amplitude modulation) fluorometer (Walz, Germany) after 10 min of plant adaptation to dark conditions. Three shoots were randomly selected from each aquarium (avoiding apical shoots due to their more active growth and photosynthesis) and measurements were obtained from the basal portion of the second youngest leaves, to minimize within-shoot variability (Durako and Kunzelman, 2002; Gera et al., 2012).

The incidence of necrosis was assessed at all leaves of five shoots from each experimental condition. Leaves were carefully separated from each shoot and the percentage of necrotic surface, considered as that partially or totally covered by dark brown or black spots, was visually estimated for each leaf and averaged for each aquarium.

Leaf growth was measured using a leaf punching method (Zieman, 1974) adapted to the model species (Pérez and Romero, 1994). At the beginning of the experiments, five shoots from each aquarium (avoiding apical shoots) were marked by punching a hole just above the ligule of the outermost leaf with a hypodermic needle. At the end of the experiments, the marked shoots were harvested, epiphytes were removed, and the leaves were carefully separated to measure leaf growth. Shoots were individually sorted into old and newly produced tissues, which were then dried for 48 h at 60 °C before obtaining their dry weights. Leaf growth rate was expressed as the new tissue produced per shoot and day (mg DW $shoot^{-1}$ $day^{-1}$), averaged for each aquarium.

To measure rhizome growth, we marked two apical shoots per aquarium with a rubber band. At the end of the experiment, these shoots were harvested, the new portions of rhizome cut, and their weight determined (after drying at 60 °C until constant). Rhizome growth was then expressed as weight of new rhizome per day (mg DW rhizome $day^{-1}$).

To estimate the shoot demographic balance (the difference between recruitment, i.e. the number of new shoots, and mortality, i.e. the number of dead shoots), all shoots surviving at the end of the experiments were counted. We computed the instantaneous demographic balance (a) as:

$$a\ (days^{-1}) = 1/t\ \ln\ (N_t/N_o)$$

Where $N_0$ is the number of shoots planted in each aquarium at the initial time (20), $N_t$ is the number of shoots alive in each aquarium at the end of each experiment and t is the duration of the experiment (in days).

Positive values for *a* occur when shoot recruitment is higher than mortality, indicating a net increase in shoot abundance. Conversely, negative values would indicate a net reduction in shoot abundance, and hence a negative response to the stressor(s) considered.

### 2.6. Statistical analysis

For all statistical analysis, an aquarium was considered as the experimental unit, with n = 3 replicates per experimental condition. The significance of the effects of temperature and ammonium, on the one hand, and, temperature and addition of labile organic C, on the other hand, were determined using PERMANOVA analysis based on a similarity matrix created from the Euclidean distances between samples. The analysis was run with two fixed factors: temperature (3 levels: 20 °C, 30 °C and 35 °C) and nutrients (3 levels: Control, Moderate and High, see above) for the TNUT experiment; and temperature (3 levels: 20 °C, 30 °C and 35 °C) and addition of labile organic C (2 levels: Control and High, see above) for the TANOX experiment.

For each experiment, one multivariate PERMANOVA was carried out for variables related to plant biochemical composition (N and S content of plant tissues, for the TNUT and TANOX experiment, respectively), and a second for the other variables ($F_v/F_m$, incidence of leaf necrosis, leaf growth, rhizome elongation, and shoot demographic balance), followed by univariate PERMANOVAs performed separately for each individual variable. As in PERMANOVA the test is produced by permutation, the usual normality assumptions of ANOVA (Anderson, 2001), that were not met by most of the variables considered, is not necessary. Pairwise comparisons were performed to identify significant differences between individual treatments. In those cases, in which the number of permutations was too low (< 999, Anderson et al., 2008), a Monte Carlo test was applied to establish an alternative p-value to validate the analysis. Analysis was carried out using the Primer v6 statistical package (Clarke and Gorley, 2006), in conjunction with the Windows PERMANOVA + module (Anderson et al., 2008).

## 3. Results

### 3.1. Culture conditions and plant biochemical composition

The different treatments (additions of nutrient and labile organic C) effectively changed the conditions under which the plants were grown. Thus, on the one hand, in the TNUT experiment, the ammonium concentrations in the water of the moderate and high treatments were increased (relative to the water in the control aquaria) to the target values at the beginning of the experiment and had decreased at the end of the experiment, despite the repeated additions of ammonium and irrespective of the thermal treatment (Table 1).

These results show that the plants were subjected at least to one strong initial pulse of ammonium, plus another two pulses during the experiment. On the other hand, in the TANOX experiment, the redox potential of the sediments at the end of the experiment, while maintaining positive values under control conditions, became negative in the mesocosms subjected to large additions of high labile organic C. Temperature affected the redox potential, with lower values at higher

**Table 1**
Ammonium concentrations (in μM, mean ± SEM, $n = 3$) in the water at the beginning (just after adding 30 μM and 300 μM to the "Moderate" and "High" treatments respectively) and at the end of the TNUT experiment.

| Ammonium treatment | Thermal treatment | | | | | |
|---|---|---|---|---|---|---|
| | 20 °C | | 30 °C | | 35 °C | |
| | $[NH4^+]$ (μM) | | | | | |
| | Initial | Final | Initial | Final | Initial | Final |
| Control | 1.20 ± 0.50 | 1.54 ± 0.53 | 0.69 ± 0.18 | 1.96 ± 1.41 | 8.64 ± 29.63 | 17.95 ± 8.29 |
| Moderate | 27.76 ± 1.12 | 0.21 ± 0.09 | 26.10 ± 0.33 | 0.62 ± 0.08 | 45.64 ± 1.30 | 3.83 ± 2.93 |
| High | 288.06 ± 34.01 | 2.21 ± 0.03 | 252.33 ± 11.23 | 4.25 ± 0.79 | 264.55 ± 7.96 | 15.82 ± 8.36 |

**Table 2**
Redox potential values (mean ± SEM, $n = 3$) of the sediment in the TANOX experiment for 7 days in three thermal treatments (20 °C, 30 °C, and 35 °C). Lower case letters indicate significant differences ($p > 0.05$) between treatments.

| Labile organic C treatment | Thermal treatment | | |
|---|---|---|---|
| | 20 °C | 30 °C | 35 °C |
| | Redox potential (mV) | | |
| Control | 180.33 ± 8.31[a] | 137.07 ± 16.41[b] | 78.59 ± 9.52[c] |
| High | −24.81 ± 6.90[d] | −230.19 ± 11.38[e] | −281.78 ± 14.84[f] |

temperatures likely due to an enhancement of the bacterial activity (Table 2).

Overall, the biochemical composition of leaves (N and S content) changed in response to the treatments. In the TNUT experiment, addition of ammonium at high concentrations increased the N content of all plant tissues, up to 23% relative to controls (Fig. 2a, b and c; Table 3). In the TANOX experiment, the S content of leaves and roots was significantly higher under conditions with an addition of labile organic C than under control conditions (Fig. 2d and f; Table 3). Temperature had significant effects on biochemical composition in both experiments. The N content of leaves increased with temperature; while the N content of rhizomes decreased at the intermediate temperature. The S content of leaves and rhizomes increased with temperature; and the latter was even higher due to interactive effects between temperature and the addition of labile organic C.

### 3.2. Effects of temperature on plant traits

Temperature had an overall significant effect on the plant traits measured in both experiments (Table 4). The maximum quantum yield of PSII ($F_v/F_m$) revealed that the photosynthetic apparatus maintained its integrity at 30 °C. However, $F_v/F_m$ was significantly depressed at 35 °C (6% and 42% lower than under control conditions, in the TANOX and TNUT experiment, respectively), suggesting that significant damage was caused by warming (Fig. 3; Table 5).

The incidence of necrosis on the leaves (Fig. 4; Table 5) was low under control conditions (20 °C; between 7% and 17% in the TANOX and TNUT experiment, respectively) and at 30 °C (< 8% in both experiments), but increased significantly to 23%–33% (depending on the experiment) at 35 °C.

Leaf growth rates (Fig. 5a and b; Table 5) showed higher values at 30 °C than under control conditions (45%) and minimum values at 35 °C (a decrease of between 63% and 94% relative to control conditions, in the TNUT and TANOX experiment, respectively). Rhizome elongation (only measured in the TANOX experiment) was also significantly higher (74%) at 30 °C than at the other two temperatures (Fig. 5c; Table 5).

The shoot demographic balance (i.e. recruitment – mortality) was clearly sensitive to temperature, with a sharp increase (83% on average, relative to the control temperature) under moderate warming (30 °C) and a clear decrease under extreme warming (35 °C), dropping to negative values in the TNUT experiment (Fig. 6a; Table 5).

### 3.3. The effects of additions of ammonium and labile organic C on plant traits

Ammonium addition did not show any effect on any of the plant traits measured (Figs. 3a, 4a and 5a and 6a; Tables 4 and 5). The addition of labile organic C did not affect the maximum quantum yield of PSII, the incidence of necrosis or the shoot demographic balance (Figs. 3b, 4b and 6b; Table 5). However, it caused a significant decrease (relative to plants grown in unaltered sediment at 30 °C) in leaf and rhizome growth rates (of 44% and 67% respectively; Fig. 5b and c; Table 5).

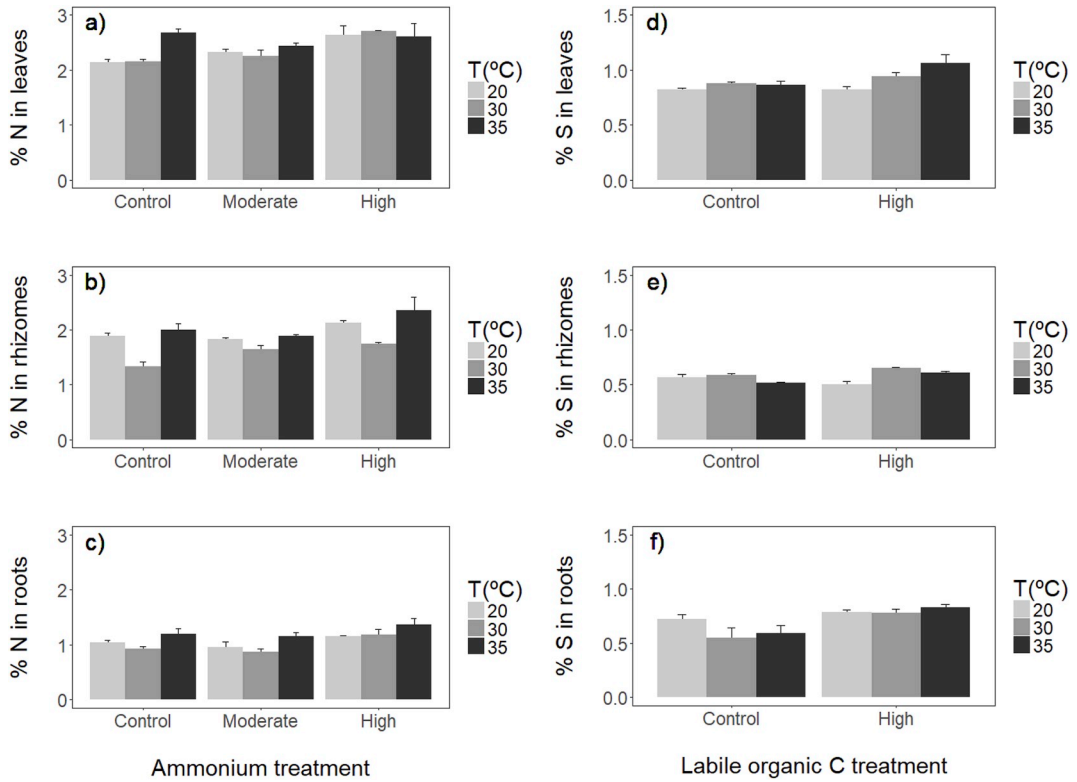


**Fig. 2.** *Cymodocea nodosa* biochemical composition (N content (mean ± SE, $n = 3$) and sulphur content (mean ± SE, $n = 3$)) measured in (a & d) roots, (b & e) rhizomes and (c & f) leaves, at 3 thermal treatments (20 °C, 30 °C, and 35 °C, light grey, dark grey and black respectively) in the TNUT (a, b & c) and TANOX (d, e & f) experiments, expressed in percentage (%).

**Table 3**
Results of PERMANOVA testing for the significance of effects of temperature (20 °C, 30 °C, and 35 °C), nutrient level (Control, Moderate, and High additions) and labile organic C addition (Control and High) on plant biochemical composition. Numbers in bold indicate significant effects ($p < 0.05$). The results of the pair-wise tests are indicated in factors with significant influence.

| Experiment | Variable | Source | df | SS | MS | Pseudo-F | Unique perms | P | Pair-wise |
|---|---|---|---|---|---|---|---|---|---|
| TNUT | | ***Main test*** | | | | | | | |
| | | Temperature | 2 | 1.802 | 0.901 | 9.839 | 9952 | **0.0001** | |
| | | Ammonium | 2 | 1.467 | 0.734 | 8.011 | 9947 | **0.0002** | |
| | | Temp x Ammonium | 4 | 0.582 | 0.145 | 1.588 | 9931 | 0.1377 | |
| | | Res | 18 | 1.648 | 0.092 | | | | |
| | N leaves | Temperature | 2 | 0.254 | 0.127 | 3.153 | 9952 | 0.0634 | |
| | | Ammonium | 2 | 0.605 | 0.302 | 7.520 | 9950 | **0.0042** | H > C = M |
| | | Temp x Ammonium | 4 | 0.373 | 0.093 | 2.317 | 9958 | 0.0955 | |
| | | Res | 18 | 0.724 | 0.04 | | | | |
| | N rhizome | Temperature | 2 | 1.257 | 0.629 | 20.145 | 9949 | **0.0001** | 35 = 20 > 30 |
| | | Ammonium | 2 | 0.594 | 0.297 | 9.519 | 9953 | **0.0012** | H > C = M |
| | | Temp x Ammonium | 4 | 0.188 | 0.047 | 1.506 | 9956 | 0.2396 | |
| | | Res | 18 | 0.562 | 0.031 | | | | |
| | N roots | Temperature | 2 | 0.291 | 0.145 | 7.219 | 9943 | **0.006** | 35 > 20 = 30 |
| | | Ammonium | 2 | 0.268 | 0.134 | 6.655 | 9945 | **0.0059** | H > C = M |
| | | Temp x Ammonium | 4 | 0.021 | 0.005 | 0.261 | 9961 | 0.9 | |
| | | Res | 18 | 0.363 | 0.02 | | | | |
| TANOX | | ***Main test*** | | | | | | | |
| | | Temperature | 2 | 25.162 | 12.581 | 9.972 | 9961 | **0.001** | |
| | | Labile organic C | 1 | 8.366 | 8.366 | 6.631 | 9952 | **0.0087** | |
| | | Temp x Lab. org. C | 2 | 7.581 | 3.790 | 3.004 | 9940 | **0.0443** | |
| | | Res | 12 | 15.140 | 1.262 | | | | |
| | S leaves | Temperature | 2 | 0.06 | 0.03 | 5.686 | 9947 | **0.0228** | |
| | | Labile organic C | 1 | 0.034 | 0.034 | 6.456 | 9823 | **0.0302** | 30 = 35 > 20 |
| | | Temp x Lab. org. C | 2 | 0.032 | 0.016 | 3.088 | 9955 | 0.0825 | C < H |
| | | Res | 12 | 0.063 | 0.005 | | | | |
| | S rhizome | Temperature | 2 | 0.021 | 0.011 | 10.223 | 9951 | **0.0012** | |
| | | Labile organic C | 1 | 0.003 | 0.003 | 3.013 | 9851 | 0.1117 | 20 = 30, 20 = 35, 30 > 35 |
| | | Temp x Lab. org. C | 2 | 0.021 | 0.01 | 9.921 | 9952 | **0.0016** | |
| | | Res | 12 | 0.012 | 0.001 | | | | |
| | S roots | Temperature | 2 | 0.026 | 0.013 | 1.435 | 9951 | 0.2638 | |
| | | Labile organic C | 1 | 0.146 | 0.146 | 16.286 | 9834 | **0.0029** | |
| | | Temp x Lab. org. C | 2 | 0.03 | 0.015 | 1.648 | 9954 | 0.2393 | C < H |
| | | Res | 12 | 0.108 | 0.009 | | | | |

### 3.4. Interactive effects

Our results did not show any significant interaction between warming and ammonium addition (TNUT experiment) in terms of their effects on plant traits. In contrast, we found interactive effects of warming and the addition of labile organic C to the sediment (TANOX experiment), both overall (Table 4) and in individual traits. Thus, the stimulation of leaf and rhizome growth at intermediate temperatures and the improvement of the shoot demographic balance were cancelled by labile organic C. In addition to this, with the normal organic C content of the sediment, high temperature (35 °C) did not alter rhizome growth or the demographic balance, but it did in the sediment with labile organic C added to it.

**Table 4**
Multivariate PERMANOVA testing for the significance of the general effect of temperature (20 °C, 30 °C, and 35 °C), nutrient level (Control, Moderate, and High additions) and labile organic C addition (Control and High) on plant traits. Numbers in bold indicate significant effects ($p < 0.05$).

| Experiment | Source | df | SS | MS | Pseudo-F | Unique perms | P |
|---|---|---|---|---|---|---|---|
| | Main test | | | | | | |
| TNUT | Temperature | 2 | 1725.100 | 862.540 | 8.612 | 9950 | **0.0030** |
| | Ammonium | 2 | 50.622 | 25.311 | 0.253 | 9949 | 0.7830 |
| | Temp x Ammonium | 4 | 144.960 | 36.241 | 0.362 | 9958 | 0.8326 |
| | Res | 18 | 1802.700 | 100.150 | | | |
| TANOX | Temperature | 2 | 105420 | 52709 | 105.350 | 9948 | **0.0001** |
| | Labile organic C | 1 | 435410 | 435410 | 870.260 | 9882 | **0.0001** |
| | Temp x Labile organic C | 2 | 25298 | 12649 | 25.282 | 9948 | **0.0001** |
| | Res | 12 | 6003.800 | 500.320 | | | |

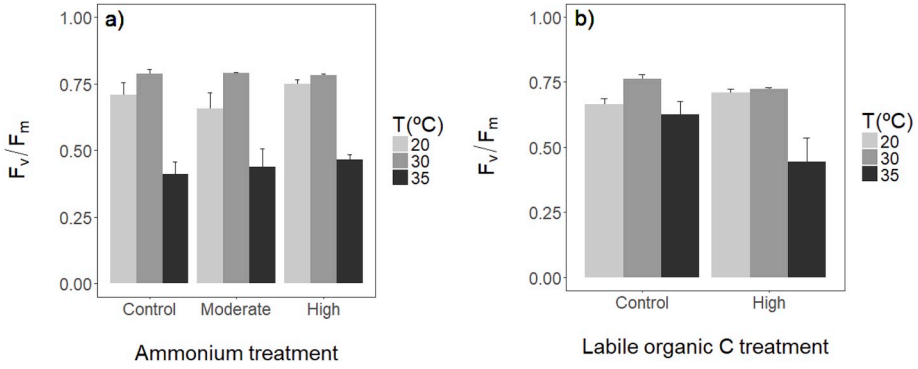


**Fig. 3.** *Cymodocea nodosa* maximum quantum yield (mean ± SE, $n = 3$) at 3 thermal treatments (20 °C, 30 °C, and 35 °C, light grey, dark grey and black respectively) in the (a) TNUT and (b) TANOX experiments.

**Table 5**
Results of PERMANOVA testing for the significance of effects of temperature (20 °C, 30 °C, and 35 °C), nutrient level (Control, Moderate, and High additions) and labile organic C addition (Control and High) on each plant trait. Numbers in bold indicate significant effects ($p < 0.05$). The results of the pair-wise tests are indicated in factors with significant influence.

| Variable | Experiment | Source | df | SS | MS | Pseudo-F | Unique perms | P | Pair-wise |
|---|---|---|---|---|---|---|---|---|---|
| Fv/Fm | TNUT | Temperature | 2 | 0.543 | 0.272 | 52.937 | 9952 | **0.0001** | 20 = 30 > 35 |
| | | Ammonium | 2 | 0.012 | 0.006 | 1.206 | 9947 | 0.3277 | |
| | | Temp x Ammo. | 4 | 0.012 | 0.003 | 0.597 | 9943 | 0.6722 | |
| | | Res | 18 | 0.092 | 0.005 | | | | |
| | TANOX | Temperature | 2 | 0.139 | 0.069 | 11.488 | 9956 | **0.001** | 30 > 20 > 35 |
| | | Labile organic C | 1 | 0.016 | 0.016 | 2.595 | 9851 | 0.1297 | |
| | | Temp x Lab. org. C | 2 | 0.04 | 0.02 | 3.283 | 9951 | 0.0555 | |
| | | Res | 12 | 0.072 | 0.006 | | | | |
| Necrosis | TNUT | Temperature | 2 | 1714.000 | 856.990 | 8.566 | 9957 | **0.0031** | 30 < 20 = 35 |
| | | Ammonium | 2 | 50.242 | 25.121 | 0.251 | 9954 | 0.7805 | |
| | | Temp x Ammonium | 4 | 144.860 | 36.215 | 0.362 | 9962 | 0.8436 | |
| | | Res | 18 | 1800.900 | 100.050 | | | | |
| | TANOX | Temperature | 2 | 4060.000 | 2030.000 | 23.683 | 9948 | **0.0006** | 20 = 30 < 35 |
| | | Labile organic C | 1 | 354.090 | 354.090 | 4.131 | 9859 | 0.0655 | |
| | | Temp x Lab. org. C | 2 | 62.753 | 31.376 | 0.366 | 9956 | 0.7057 | |
| | | Res | 12 | 1028.600 | 85.714 | | | | |
| Leaf growth rate | TNUT | Temperature | 2 | 10.538 | 5.269 | 53.080 | 9950 | **0.0001** | 30 > 20 > 35 |
| | | Ammonium | 2 | 0.367 | 0.183 | 1.848 | 9950 | 0.186 | |
| | | Temp x Ammonium | 4 | 0.091 | 0.023 | 0.228 | 9951 | 0.9224 | |
| | | Res | 18 | 1.787 | 0.099 | | | | |
| | TANOX | Temperature | 2 | 12.713 | 6.357 | 54.210 | 9955 | **0.0001** | 30 > 20 > 35 |
| | | Labile organic C | 1 | 1.070 | 1.070 | 9.125 | 9851 | **0.0058** | C > H |
| | | Temp x Lab. org. C | 2 | 1.327 | 0.663 | 5.657 | 9951 | **0.0075** | |
| | | Res | 12 | 1.407 | 0.117 | | | | |
| Rhizome growth rate | TANOX | Temperature | 2 | 38.329 | 19.164 | 15.536 | 9954 | **0.0011** | 30 > 20 = 35 |
| | | Labile organic C | 1 | 23.510 | 23.510 | 19.059 | 9739 | **0.0012** | C > H |
| | | Temp x Lab. org. C | 2 | 22.905 | 11.452 | 9.284 | 9948 | **0.0035** | |
| | | Res | 12 | 14.803 | 1.234 | | | | |
| Shoot demographic balance | TNUT | Temperature | 2 | 0.005 | 0.003 | 33.785 | 9964 | **0.0001** | 30 > 20 > 35 |
| | | Ammonium | 2 | 0.001 | 0.001 | 1.097 | 9957 | 0.3624 | |
| | | Temp x Ammonium | 4 | 0.001 | 0.001 | 0.152 | 9962 | 0.9594 | |
| | | Res | 18 | 0.001 | 0.001 | | | | |
| | TANOX | Temperature | 2 | 0.001 | 0.001 | 6.767 | 9952 | **0.0119** | 30 > 20 = 35 |
| | | Labile organic C | 1 | 0.001 | 0.001 | 3.571 | 9758 | 0.0887 | |
| | | Temp x Lab. org. C | 2 | 0.001 | 0.001 | 5.231 | 9959 | **0.0219** | |
| | | Res | 12 | 0.001 | 0.001 | | | | |

## 4. Discussion

Climate change is having an impact on a world that has already been altered by a panoply of local stressors. Our results show how cumulative stress, in this case derived from the joint action of warming and eutrophication, can worsen, either through additive or interactive effects, the negative consequences of each stressor acting in isolation.

As already known, temperature increases can negatively affect different functional mechanisms of seagrasses (Koch et al., 2013). The response patterns and the thresholds involved are largely species

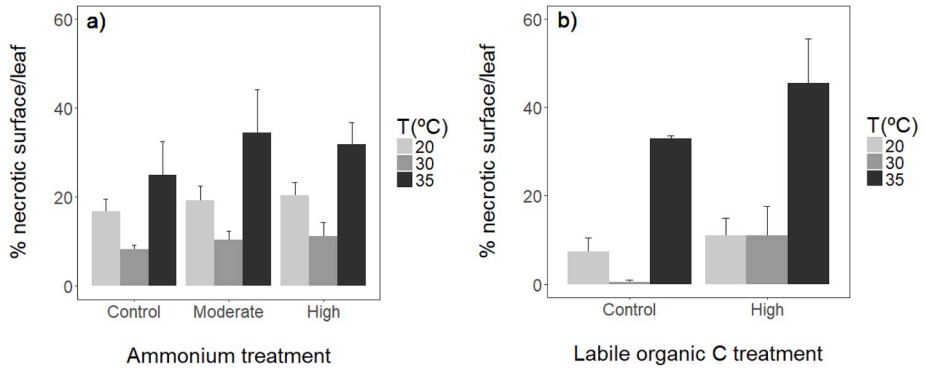


**Fig. 4.** Leaf necrosis incidence (average necrotic surface per leaf, in %) in *Cymodocea nodosa* (mean ± SE, $n = 3$) at 3 thermal treatments (20 °C, 30 °C, and 35 °C, light grey, dark grey and black respectively) in the (a) TNUT and (b) TANOX experiments.

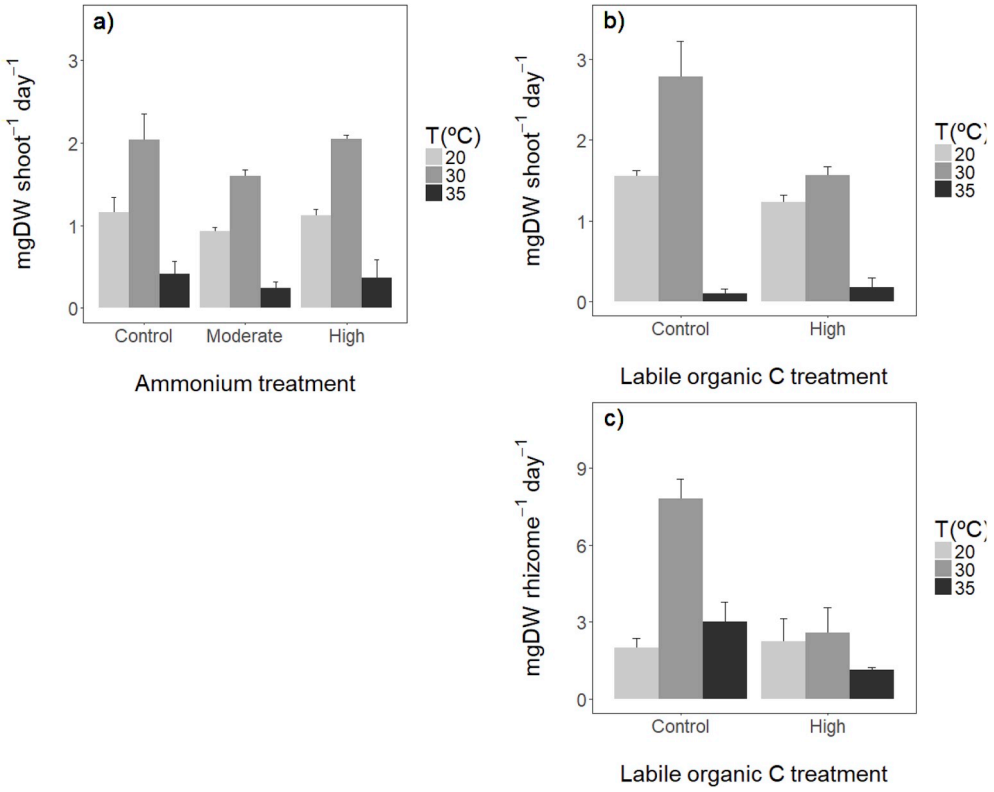


**Fig. 5.** *Cymodocea nodosa* growth rate (mean ± SE, $n = 3$) at 3 thermal treatments (20 °C, 30 °C, and 35 °C, light grey, dark grey and black respectively) in two experiments: (a) Leaf growth rate in the TNUT experiment; (b) Leaf growth rate in the TANOX experiment; (c) Rhizome growth rate in TANOX experiment.

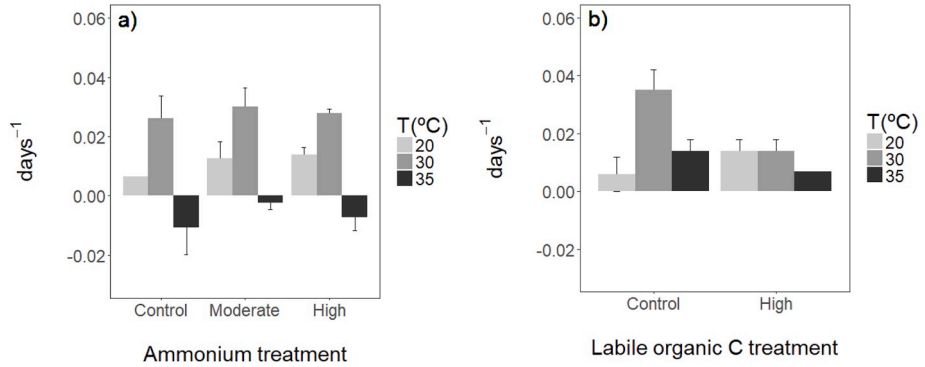


**Fig. 6.** *Cymodocea nodosa* shoot demographic balance (mean ± SE, $n = 3$) at 3 thermal treatments (20 °C, 30 °C, and 35 °C, light grey, dark grey and black respectively) in the (a) TNUT and (b) TANOX experiments.

specific (Campbell et al., 2006; Collier et al., 2011). In the model species used here (*C. nodosa*), moderate warming seems to be beneficial for the plant, whose performance (photosynthesis, growth and shoot demographic balance) increase at 30 °C, relative to those found at the basal spring temperature (control) of 20 °C. This pattern is fully consistent in the two experiments we conducted. This is in accordance with previous reports for this species, suggesting an optimum temperature close to 30 °C (Olsen et al., 2012; Pérez and Romero, 1992; Savva et al., 2018; Terrados and Ros, 1995; Tutar et al., 2017). In contrast, plant performance was severely depressed at 35 °C; not only relative to the 30 °C optimum, but also relative to control conditions. This suggests there is a thermal threshold of clear negative effects on plant activity between 30 °C and 35 °C. This thermal threshold is relatively high (see Lee et al., 2007 for comparisons), and it is in accordance with the subtropical distribution of this species (Green and Short, 2003; Reyes et al., 1995) and its facultative habitat in confined environments, where summer temperatures can easily be 5 °C above open sea temperatures.

Exposure to extreme thermal values damaged the integrity of the photosynthetic apparatus, as shown by a clear drop in $F_m/F_v$ to values below those considered acceptable for healthy plants (0.7–0.8, Campbell et al., 2006; Ralph, 1998), as previously found for other seagrass species (e.g. *T. testudinum*; Koch et al., 2007; e.g. *Z. noltii;* Massa et al., 2009). While photosynthesis is depressed, respiration is probably stimulated by thermal stress (not measured in this study; but see Pérez and Romero, 1992), leading to impairment of the C budget (Collier and Waycott, 2014), which could be the cause of the reduced growth and the low to negative shoot demographic balance observed in our experiments. Plants exposed to high temperatures may have to use their energy reserves (stored non-structural carbohydrates) to cope with this stress and the consequent energy requirement (Collier et al., 2011; Massa et al., 2009), probably leading to exhaustion of the internal C reserves (Marín-Guirao et al., 2018). Indeed, thermal stress also affects other metabolic processes, causing, for instance, oxidative stress (Tutar et al., 2017), and ultimately affecting plant health, which deteriorated in our experiments as shown by the increase in the incidence of necrosis.

Reducing the shoot demographic balance can be critical for *C. nodosa*. This species has a very high shoot turnover, with a yearly shoot mortality reaching 1/2 to 2/3 of the total number of shoots in unaltered meadows. This mortality takes place in late summer to autumn and is balanced by massive recruitment in late spring (Mascaró et al., 2014). Any event altering the shoot demographic balance, such as a heat wave, will cause a drop in seagrass density, eventually leading to meadow extinction. This is relevant for projections of distribution and abundance of this species in future warming scenarios since the frequency and intensity of heat waves are predicted to increase (IPCC, 2013). Those predictions suggest that the threshold temperature (thermal tolerance limit) could be reached during these extreme climate events, mainly in confined areas such as shallow bays or lagoons. However, the threshold is quite unlikely to be reached in the open sea, where warming will be much more moderate and could have beneficial effect on the species which could extend its distribution, maybe at the expenses of the Mediterranean species *P. oceanica*, which is much more sensitive to warming (Marín-Guirao et al., 2016; Olsen et al., 2012).

Regarding eutrophication, *C. nodosa* is affected by an increase in organic matter in the sediment but not by pulses in nutrient concentrations. None of the traits studied were modified by addition of ammonium, despite the high concentrations attained (up to 300 μM) and the fact that ammonium was depleted from the aquaria. A coarse N mass balance, estimating N incorporation in the plant through leaf growth, new shoots and N increase in tissues, suggests that most of this depletion was caused by plant activity, being microbial activity in the sediment the most likely explanation for the rest. Seagrasses seem to be unable to downregulate N uptake, probably due to a lack of inhibitory feedback mechanisms (Touchette and Burkholder, 2000). This failure in regulation could generate ammonium accumulation in cells, which in turn may have toxic effects (Invers et al., 2004). However, while some species seem to be more vulnerable to this toxicity (e.g. *Z. marina*, Burkholder et al., 1992; Van Katwijk et al., 1997; Villazán et al., 2013; and *Z. noltei*, Moreno-Marín et al., 2016) others show great resistance (*C. nodosa*, Egea et al., 2018 and *Z. marina*, Kaldy, 2014). It has been suggested that the key mechanism to endure large ammonium pulses may be an efficient mechanism that is capable of rapidly converting the excess of ammonium into organic forms (Brun et al., 2002; Invers et al., 2004). Second-order (indirect) effects of ammonium pulses, such as an increase in epiphytic load or a decrease in water transparency, were not studied here and cannot be ruled out.

In contrast, the addition of labile organic C had a detrimental effect on plants. The organic additions to the sediment seemed to enhance bacterial respiration and thus oxygen demand, leading to oxygen exhaustion and anoxic conditions (up to −290 mV of redox potential). Under these conditions, sulphate reduction is stimulated, resulting in sulphide accumulation. Consistently with this, we found higher sulphur contents in our exposed plants than in controls. The oxidation of sulphide to sulphur compounds that are further stored in tissues has been shown to be a mechanism that can help cope with sulphide intrusion. However, once the capacity of detoxification of this mechanism is surpassed, the detrimental effects appear (Hasler-Sheetal and Holmer, 2015). Although *C. nodosa* is highly resistant to eutrophication (Oliva et al., 2012), highly negative values (such as those created in our experiment, close to −250 mV) clearly seem to be harmful for plant production and fitness.

Beyond the effects of warming and eutrophication highlighted above, and given that both stressors will act jointly in most real-world conditions, the assessment of their potential interactions is of great interest. Our results show that there were no interactive effects between warming and ammonium; but in contrast the effects of warming on key processes (leaf and rhizome growth and the demographic balance) were strongly mediated by the amount of labile organic C in the sediment. The interaction between temperature and organic matter was detected at the individual (leaf and rhizome growth) and population (shoot demographic balance) level, but not at the physiological one ($F_v/F_m$). Clearly, the processes affected are critical for meadow persistence, which underlines the relevance of such interactions for the prediction of future seagrass meadow dynamics. However, the mechanisms through which these interactions function have not been elucidated by our work. A possible explanation would be a synergistic effect on environmental conditions. This is supported by the fact that the addition of labile organic C and temperature decreased sediment redox potential synergistically, probably through the stimulation of oxygen demand and cascading effects on sulphide production and plant performances. Other mechanisms, including the amplification of sulphide effects by temperature, should not be ruled out.

Despite multiple stressors studies have increased in the last decades, our results add evidence to the need to further assess the interactive effects of different stressors, and understanding how the organisms or communities will respond to the impact of multiple co-ocurrent stressors is still a matter of concern (Côté et al., 2016). Seemingly, synergistic effects are quite frequent, as revealed by Crain et al. (2008), which found in a review focused on coastal ecosystems that 36% of the cases examined showed synergy. A thorough literature search on interactive effects on seagrass ecosystems (Table 6) confirms that synergy is more the rule (50%) than the exception (36% additive; only a small part of the studies found antagonistic interaction). There is an urgent need to incorporate those interactive effects to improve predictions of the consequences of climate change in marine ecosystems, which can be seriously underestimated when assessing thermal effects in isolation. In addition, results such as those presented here can support strategies to increase ecosystem resilience to climate change by managing other stressors at a local or regional scale. In this respect, shallow bays and coastal lagoons, which are more vulnerable to both extreme thermal events and eutrophication, may represent a critical scenario for the

**Table 6**
Synthesis of multiple stressors studies on seagrasses and the interaction type effects.

| Stressor 1 | Stressor 2 | Stressor 3 | Overall interaction type | Species | Source |
|---|---|---|---|---|---|
| Temperature | Nitrate | | Additive | *Zostera marina* | Kaldy (2014) |
| Temperature | Ammonium | | Additive | *Cymodocea nodosa* | This study |
| Temperature | Labile Organic C | | Synergy | *Cymodocea nodosa* | This study |
| Temperature | Acidification | | Additive | *Zostera noltii* | Repolho et al. (2017) |
| Temperature | Herbicide | | Antagonism | *Halophila ovalis* | Wilkinson et al. (2017) |
| Temperature | Light | | Additive | *Zostera muelleri* | York et al. (2013) |
| Temperature | Light | | Synergy | *Halodule uninervis* and *Zostera muelleri* | Collier et al. (2011) |
| Temperature | Floods | | Synergy | *Amphibolis antarctica* | Fraser et al. (2014) |
| Temperature | Macroalgae | | Synergy | *Zostera marina* | Höffle et al. (2011) |
| Temperature | Salinity | | Synergy | *Zostera marina* | Salo and Pedersen (2014) |
| Temperature | Sulfide | | Synergy | *Halodule wrightii* and *Thalassia testudinum* | Koch et al. (2007) |
| Nitrate | Acidification | | Additive | *Thalassia testudinum* | Campbell and Fourqurean (2014) |
| Nitrate | Acidification | | Additive | *Thalassia hemprichii* and *Halodule uninervis* | Ow et al. (2016) |
| Nitrate and phosphate | Acidification | | Additive | *Thalassia testudinum* | Campbell and Fourqurean (2018) |
| Nitrate, ammonium and phosphate | Waves | | Synergy | *Zostera noltii* | La Nafie et al. (2012) |
| Ammonium | Light | | Synergy | *Zostera marina* | Villazán et al. (2013) |
| Ammonium | Macroalgae | | Antagonism | *Zostera noltii* | Moreno-Marín et al. (2016) |
| Ammonium | Salinity | | Synergy | *Zostera marina* | Villazán et al. (2015) |
| Organic matter | Burial | | Synergy | *Posidonia oceanica* | Ceccherelli et al. (2018) |
| Temperature | Ammonium | Light | Synergy | *Zostera marina* | Moreno-Marín et al. (2018) |
| Temperature | Ammonium | Acidification | Additive/Synergy | *Cymodocea nodosa* | Egea et al. (2018) |
| Ammonium | Phosphate | Light | Antagonism | *Zostera noltii* | Brun et al. (2008) |

survival of seagrass species growing close to their upper thermal limit (York et al., 2013; Koch et al., 2007), but also an opportunity to test the above mentioned strategies.

Even though our findings, it is important to keep in mind that the results of this work were obtained from a mesocosm experiment focusing only on two factors (warming and eutrophication) without considering any other disturbance that may be found in the environment. In this sense, they should only be extrapolated to natural conditions cautiously. In spite of these limitations, this research highlights the importance of evaluating the impact of global and local stressors jointly; not only to generate more realistic predictions of the impacts that climate change might have, but also to design and implement strategies to improve (or at least not to impair) seagrass resilience to global warming.

## Author contributions

YO, EG, NS, JMR, JR and MP planned and designed the study. YO, EG and NS conducted fieldwork, performed the experiments, samples processing and lab work. YO and JB analyzed the data. YO, JR and MP led the writing of the paper with contributions from the rest of authors. All Authors reviewed the manuscript.

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